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Dysregulation of survival and apoptosis pathways in acute myeloid leukemia

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Bosman, M. C. J. (2015). *Dysregulation of survival and apoptosis pathways in acute myeloid leukemia: Targeting of NF- κ B in AML*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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CHAPTER

7

SUMMARY AND DISCUSSION

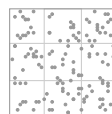


7.1 SUMMARY

Leukemia development is a multi-step process in which genetic aberrations have an effect on several biological processes such as self-renewal, differentiation and apoptosis, which results in the preservation and expansion of the (pre)-malignant cells. Understanding the differences in biological mechanisms of AML stem cells compared to normal hematopoietic stem cells might result in the identification of novel targets for the treatment of AML. The NF- κ B pathway has emerged as an important signaling pathway in AML which regulates the survival of AML (stem) cells and mediates chemoresistance. Constitutive activation of NF- κ B is frequently observed in AML and it has been proposed that targeting of NF- κ B is a potential treatment strategy in these patients.

In **Chapter 2** we described the various molecular mechanism that result in the constitutive activation of NF- κ B. In the last decade a number of studies have shown that an initial chromosomal translocation or mutation can directly lead to enhanced activation of NF- κ B. Moreover, the expression of cytokines regulating NF- κ B activity, such as TNF α and IL-1, is often increased. Recent data also indicate that the expression of multiple positive regulators of NF- κ B activity is frequently increased. Lastly, enhanced proteasome expression and activity has been detected in AML cells. As NF- κ B activity is controlled by the proteasome via degradation of the negative regulator I κ B α , increased proteasome activity could result in constitutive NF- κ B activation. All these factors might cooperate resulting in sustained NF- κ B activation. Based on these different mechanisms, various treatment strategies have been proposed that result in the inhibition of NF- κ B activity and thereby induce cell death. We have studied two of these strategies in detail: proteasome inhibition (**Chapter 3** and **5**) and targeting of TAK1 (**Chapter 6**). The proteasome inhibitor bortezomib has emerged as successful strategy for the treatment of multiple myeloma patients. In **Chapter 3** we have verified the sensitivity of primary stem cell-enriched (CD34⁺) AML cells to bortezomib alone or in combination with TRAIL. We observed that AML CD34⁺ cells were less sensitive to bortezomib than AML CD34⁻ cells. Co-inhibition of AML cells with TRAIL did not enhance the cytotoxic effect of bortezomib in contrast to the cell death inducing effects that we and others observed in AML cell lines. Resistance to bortezomib of AML CD34⁺ was mediated by the persistent activity of NF- κ B and upregulation of the anti-apoptotic protein MCL-1 in these cells. Co-treatment of bortezomib with the IKK inhibitor BMS-345541 resulted in additive cytotoxic effects. Furthermore, genetic or chemical targeting of MCL-1 significantly enhanced the sensitivity of AML cells to bortezomib, indicating that the co-treatment with MCL-1 inhibitors could potentially result in the eradication of AML cells.

The bone microenvironment interacts with (malignant) hematopoietic cells via direct and indirect contact. Various cytokines, secreted by the bone microenvironment, could not only induce the proliferation and survival of malignant cells but also mediate chemoresistance. OPG is secreted by osteoblasts and could possibly prevent TRAIL-induced cell death by acting as a decoy receptor. Therefore, we investigated whether previously designed death receptor-specific variants could



overcome TRAIL resistance in multiple myeloma cells mediated by OPG (**Chapter 4**). We showed by surface plasmon resonance and ELISAs that a DR5-specific variant (D269H/E195R) has a significantly lower affinity for OPG than wild-type TRAIL. Moreover, the cytotoxic activity of this variant was increased compared to wild-type TRAIL and the activity was retained in the presence of OPG secreted by stromal cells. These data indicate that this variant could be used as a novel strategy for the treatment of multiple myeloma and other malignancies.

In **Chapter 5** we have studied whether the second-generation proteasome inhibitors carfilzomib and oprozomib had an improved effectiveness to induce cell death in primary AML cells compared to bortezomib. Treatment of AML CD34⁺ cells with the proteasome inhibitors in long-term culture conditions showed that carfilzomib had an improved efficacy compared to bortezomib or oprozomib treatment. This coincided with a reduction in quiescent AML CD34⁺CD38⁻ cells. The higher efficacy of carfilzomib compared to bortezomib could be due to differences in the mode of action of both inhibitors. Whereas bortezomib binds reversibly to the proteasome, carfilzomib binds irreversibly to the proteasome and could potentially inactivate the proteasome for a longer period. We showed that carfilzomib indeed reduced the proteasome activity for a longer period suggesting that increased efficacy of carfilzomib is due to a difference in binding to the proteasome. Importantly, normal bone marrow cells were less affected upon carfilzomib treatment. We observed that expression of proteasome subunits as well as the proteasome activity was increased in AML CD34⁺ cells, which could possibly explain the difference in sensitivity. MCL-1 upregulation could mediate resistance to proteasome inhibitors, as has been observed in **Chapter 2**. Addition of carfilzomib to AML cells also resulted in an upregulation of MCL-1 expression. Co-inhibition with the MCL-1 inhibitor obatoclax significantly improved the efficacy of carfilzomib, suggesting that the combination of carfilzomib with MCL-1 might be more effective to eradicate AML LSCs.

In **Chapter 6** we showed that the expression of apoptosis-associated genes is different in AML CD34⁺ cells compared to normal bone marrow CD34⁺ cells suggesting that AML cells differently depend on apoptotic signaling. TAK1 overexpression was verified by QPCR and western blot and demonstrated that TAK1 is frequently upregulated in AML CD34⁺ cells. Targeting of TAK1 by genetic downmodulation or chemical inhibition with the TAK1 inhibitors 5z-7-oxozeaenol and AZ-TAK1 induced cell death in AML cell lines and primary AML CD34⁺ cells in vitro. Moreover, downmodulation of TAK1 resulted in impaired development of leukemia in vivo and improved survival of mice in a humanized xenograft model. We further showed that repression of NF- κ B is an important mechanism for the cytotoxic effects observed upon inhibition of TAK1. These data indicate that TAK1 activity is required for the maintenance of AML. In contrast, TAK1 is most likely not involved in the initiation process of leukemia as overexpression of TAK1 did not result in myeloid transformation. Nevertheless, these experiments suggest that targeting of TAK1 could be a potential strategy for the treatment of AML patients.



7.2 DISCUSSION

Leukemic stem cells play a central role in the development and maintenance of acute myeloid leukemia. At present, many studies focus on the identification of the various (pre-) leukemic stem cell populations in AML patients by e.g. next-generation sequencing, and the differences in responses of these subpopulations to chemotherapy. These studies are necessary to elucidate which subpopulations are resistant to the current therapy and eventually evoke relapse in patients. At the same time, research intends to characterize these specific subpopulations of leukemic stem cells. Studying the regulation of cellular programs such as apoptosis, autophagy, metabolism and membrane receptor expression in AML, and to what extent leukemic cells differently depend on these biological programs compared to normal hematopoietic stem cells, is necessary to discover potential new targets for the treatment of AML patients. As resistance to apoptosis is a common hallmark during the development and maintenance of cancer, our research described in this thesis has focused on the difference in regulation of the apoptotic machinery in normal and leukemic cells. The NF- κ B pathway is one of the pro-survival mechanisms that is constitutively activated in a large subset of AML patients. Furthermore, repression of NF- κ B specifically induced cell death in AML cells, whereas normal hematopoietic cells were relatively insensitive. In this thesis we investigated various approaches to eradicate AML cells by the inhibition of NF- κ B. Inhibitors of NF- κ B could have a broad application as a large frequency of patients has increased NF- κ B activity. We have shown that the second generation proteasome inhibitor carfilzomib triggers apoptosis in primitive AML CD34⁺ cells, in contrast to the first generation inhibitor bortezomib. It is however unclear whether this cytotoxic effect is partially mediated via the blockade of the NF- κ B pathway. Future experiments will therefore be required to evaluate whether carfilzomib reduces NF- κ B activity in AML cells.

To predict clinical outcome, it is necessary to find relationships between response to chemotherapy and biological or clinical characteristics. At the moment, our data indicate that the sensitivity to carfilzomib is not related to the chymotrypsin-like activity of AML cells or patient characteristics. Further studies are necessary to determine whether there are any relationships between the sensitivity to carfilzomib of AML cells to any other biological characteristics, such as the level of NF- κ B activity.

The effectiveness of carfilzomib has been investigated in short- and long-term experiments *in vitro*. To further evaluate whether carfilzomib induces apoptosis in AML cells, it will be necessary to investigate the effect of carfilzomib on primary AML cells *in vivo*. Furthermore, the combination of carfilzomib with MCL-1 inhibitors should be investigated to overcome the resistance to carfilzomib mediated by the proteasomal-dependent upregulation of MCL-1.

Similarly, the effectiveness of TAK1 inhibition on primary AML stem cells should be studied in *in vivo* experiments. We have already evaluated the effect of TAK1 knockdown on primary AML CD34⁺ cells *in vivo*. We transplanted patient-derived AML CD34⁺ cells with shSCR or shTAK1 mCherry⁺ cells into our humanized

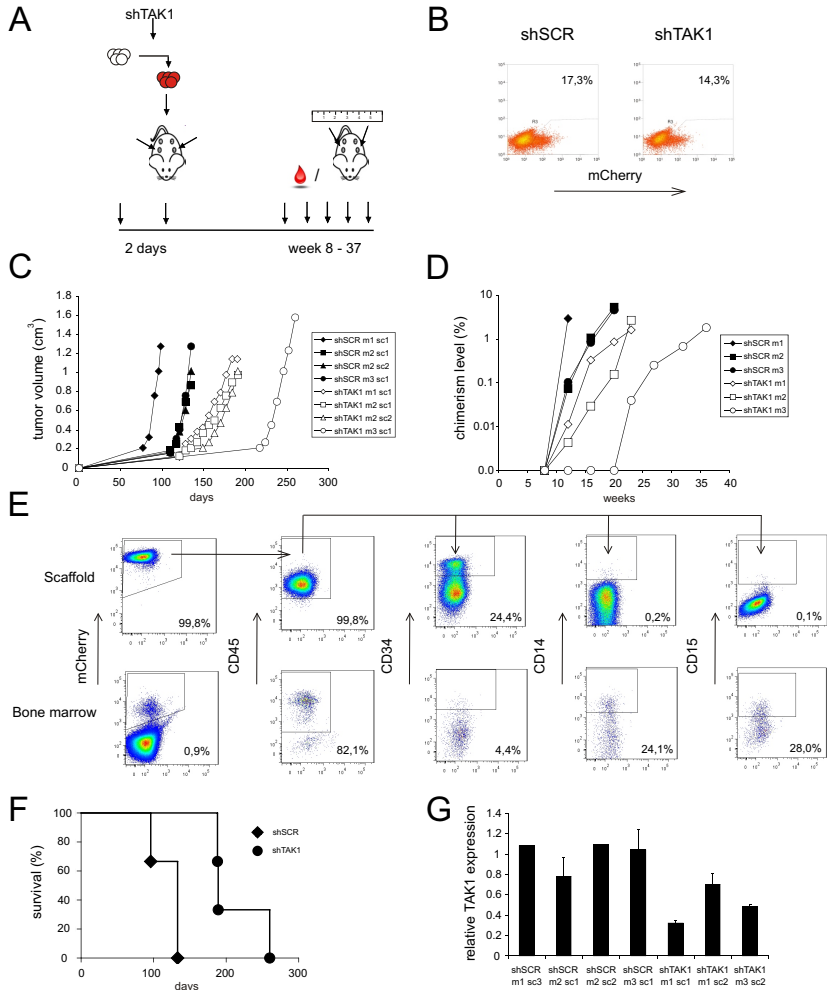
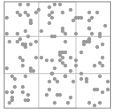


Figure 7.1: TAK1 downmodulation in AML cells results in delayed leukemia development and increased survival in a humanized scaffold niche xenograft model

A) Experimental set-up: AML CD34⁺ cells were transduced with pLKO.1 shSCR mCherry or shTAK1 mCherry and 2 days later, mCherry positive cells were sorted and injected intrascapularly. Leukemia progression was determined by measuring the tumor volume and chimerism level in peripheral blood B) Sorting strategy of AML pLKO.1 shSCR mCherry and shTAK1 mCherry positive cells C) Tumor growth of shSCR and shTAK1 mice D) Chimerism level in peripheral blood of shSCR and shTAK1 mice E) Example of CD45/CD34/CD14/CD15 levels in scaffold and bone marrow F) Kaplan-Meier curve of shSCR and shTAK1 mice G) Expression levels of TAK1 in shSCR and shTAK1 mice determined by Q-RT-PCRs



scaffold niche xenograft model (Figure 7.1A/B). In this model a human niche is created by the transplantation of ceramic scaffolds coated with human mesenchymal stromal cells on the back of the mice. Time of onset of leukemia development and tumor growth was significantly delayed upon TAK1 knockdown as measured by tumor volume (Figure 7.1C) and peripheral blood chimerism level (Figure 7.1D). Upon sacrifice, myeloid leukemia cells (mCherry⁺/CD45⁺/CD33⁺) were not only observed within the scaffolds but infiltrations were also observed in the murine bone marrow (chimerism level = $3.4\% \pm 1.3\%$), spleen ($26.2\% \pm 18.3\%$), and liver ($8.3\% \pm 5.9\%$), in both the shSCR mice as well as the shTAK1 transplanted mice. However, the human leukemic cells within the murine bone marrow seemed to be more differentiated with a significantly lower frequency of CD34⁺ cells ($20.7\% \pm 11.5\%$ versus $28\% \pm 6\%$, $p < 0.01$) and a significantly higher frequency of CD14⁺ ($24.4\% \pm 13.0\%$ versus $6.6\% \pm 4.7\%$, $p < 0.01$) and CD15⁺ cells ($20.2\% \pm 10.1\%$ versus $3.8\% \pm 4.5\%$, $p < 0.01$) (Figure 7.1E), as has been observed previously (unpublished data, P. Sontakke et al). Kaplan Meier curves showed that survival of shTAK1 transplanted mice was significantly improved compared to shSCR transplanted mice (Figure 7.1F). Efficiency of TAK1 knockdown at sacrifice of some of the shTAK1 mice was lower than 50% suggesting that leukemic cell growth was caused by a selection of clones with an inefficient knockdown of TAK1 (Figure 7.1G). However, it cannot be excluded that other survival pathways were activated and compensated the loss of TAK1 in these cells.

Nevertheless, these data indicate that targeting of TAK1 abrogates the growth of primary myeloid leukemic cells *in vivo*. Based on these results, it will be important to evaluate the effect of TAK1 inhibitors on the growth of primary AML cells *in vivo*. Whereas the effects of the TAK1 inhibitors 5z-7-oxozeaenol and AZ-TAK1 could not be studied *in vivo* due to their low solubility or pharmacokinetic properties, a novel TAK1 inhibitor has recently been described which can be used in *in vivo* experiments [1]. It will be interesting to evaluate the effect of this inhibitor on the growth and maintenance of primary AML stem cells *in vivo*.

In our studies we have observed heterogeneity in response of patient AML cells to the TAK1 inhibitors. It should be noted that the difference in sensitivity between these cells is not related to the expression level of TAK1. Nevertheless, protein expression levels and protein activity are two different entities and it might be possible that the response to TAK1 inhibiting agents is correlated with the activity of TAK1. Until now, we have not been able to determine TAK1 activity in AML cells, but it is still worth pursuing these experiments by e.g. phosphoproteomics, as TAK1 activity levels might predict the clinical response to TAK1 inhibitors.

Another important remaining issue is understanding the exact downstream signaling pathways that are involved upon inhibition of TAK1. We have observed that the cytotoxic effects upon TAK1 inhibition are partially mediated by repression of NF- κ B. However, we could not exclude the possibility that other pathways downstream of TAK1 are also affected. Recently, it has been suggested that simultaneous inhibition of both NF- κ B and JNK induces a synergistic cytotoxic effect in AML cells and in particular in AML LSCs [2]. As both pathways are activated by TAK1, we also questioned whether repression of the TAK1-JNK axis contributes to

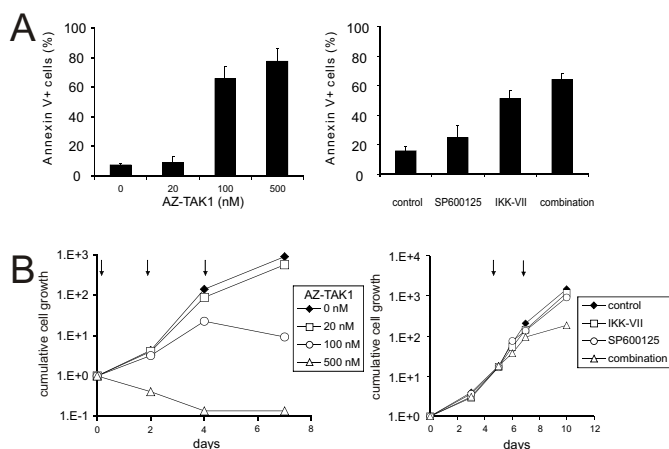


Figure 7.2: Cell death induced upon TAK1 inhibition is mainly mediated by repression of NF- κ B and JNK activity

A) CB MLL-AF9 cells were cultured in liquid in the presence of IL-3, FLT3L as well as CSF and treated for 24 hrs with various concentrations of AZ-TAK1, 2 μ M of IKK-VII, 10 μ M of SP600125 or the combination of 2 μ M of IKK-VII and 10 μ M of SP600125 and apoptosis was determined by Annexin V staining B) CB MLL-AF9 cells were grown on MS5 bone marrow stromal cells in the presence of IL-3, FLT3L as well as CSF and at the indicated time points (\downarrow), cells were treated with various concentrations of AZ-TAK1, 2 μ M of IKK-VII, 10 μ M of SP600125 or the combination of 2 μ M of IKK-VII and 10 μ M of SP600125 and cumulative cell growth was determined

the cytotoxic effects observed upon TAK1 inhibition. To answer this question we used the MLL-AF leukemia model in which we treated MLL-AF9 transduced cord blood cells for 24 hrs in liquid culture with the TAK1 inhibitor 5z-7-oxozeanol. Inhibition of TAK1 resulted in apoptosis (Figure 7.2A). The NF- κ B inhibitor also induced cell death in MLL-AF9 cord blood cells, but only mild cytotoxic effects were observed upon JNK repression by SP600125. These findings are in line with the results obtained when AML cell lines were treated with NF- κ B or JNK inhibitors (**Chapter 6**). Co-addition of IKK-VII and SP600125 had a small, but significant additive effect in CB MLL-AF9 cells, consistent with the data observed by Volk et al. [2]. In contrast, when cells were treated on a MS5 stromal layer by IKK-VII or SP600125 no cytotoxic effect was observed (Figure 7.2B). These data suggested that the micro-environment has a protective role on the survival of AML cells as has also been observed with other agents, such as FLT3-ITD inhibitors. However, a synergistic cytotoxic effect was observed when CB MLL-AF9 cells were treated with the combination of NF- κ B and JNK inhibitors for a long period on the MS5 stromal layer (Figure 7.2B). This suggests that the microenvironment induces NF- κ B and/or JNK activity thereby mediating resistance to IKK-VII or SP600125. However,



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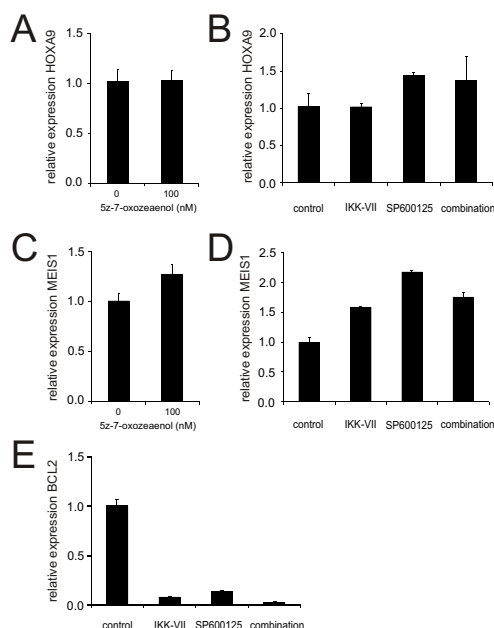


Figure 73: TAK1 inhibition results via inhibition of NF- κ B and JNK in repression of BCL2 expression, whereas HOXA9 and MEIS1 expression are unaffected

A) THP1 cells were treated for 6 hours with 100 nM of 5z-7-oxozeaeenol and HOXA9 levels were determined B) CB MLL-AF9 cells were cultured in liquid in the presence of IL-3, FLT3L as well as CSF and treated for 6 hours with 2 μ M of IKK-VII, 10 μ M of SP600125 or the combination of 2 μ M of IKK-VII and 10 μ M of SP600125 and HOXA9 levels were determined C) THP1 cells were treated for 6 hours with 100 nM of 5z-7-oxozeaeenol and MEIS1 levels were determined D) CB MLL-AF9 cells were cultured in liquid in the presence of IL-3, FLT3L as well as CSF and treated with 2 μ M of IKK-VII, 10 μ M of SP600125 or the combination of 2 μ M of IKK-VII and 10 μ M of SP600125 and MEIS1 levels were determined E) CB MLL-AF9 cells were cultured in liquid in the presence of IL-3, FLT3L as well as CSF and treated with 2 μ M of IKK-VII, 10 μ M of SP600125 or the combination of 2 μ M of IKK-VII and 10 μ M of SP600125 and BCL2 levels were determined

this protective role is lost upon simultaneous repression of NF- κ B and JNK which might result in a synergistic cytotoxic effect. Furthermore, co-inhibition of both pathways largely mimicked the effects seen when the MLL-AF9 cells were treated with AZ-TAK1, suggesting that cell death mediated by TAK1 inhibition is mainly mediated by repression of both NF- κ B and JNK.

Recently, it was proposed that NF- κ B can also directly be involved in the process of leukemic transformation by recruiting MLL oncoproteins to the HOXA9 and MEIS1 promoters thereby regulating the expression of MLL target genes [3]. These data suggest that cell death mediated via NF- κ B repression would occur, at least



in part, via inhibition of MLL target genes rather than via loss of induction of NF- κ B target genes. Since these published experiments were performed using MLL-transformed murine cells, we wondered whether NF- κ B would also directly be involved in the regulation of transcription of MLL-target genes in a human background. In contrast to the results by Kuo et al., we did not observe a repression of HOXA9 and MEIS1 expression upon inhibition of TAK1 or NF- κ B, whereas the expression of BCL-2 was largely inhibited (Figure 7.3). These data indicate that apoptosis induced by TAK1 or NF- κ B inhibition is not mediated via repression of the MLL target genes but by direct inhibition of the survival machinery. To verify these results ChIP experiments with p65 antibodies have to be performed to show the direct binding of p65 on the HOXA9 and MEIS1 promotor sites. It has further been suggested that BRD4, a member of the bromodomain and extraterminal domain (BET) family, binds to acetylated p65 and enhances the transcriptional activation of p65 [4]. BRD4 has emerged as important target in AML as inhibition of BRD4 by short hairpins or inhibitors, such as JQ1 and IBET-151, resulted in cell death of primary AML cells and prolonged the survival of mice in various MLL-oncoprotein models [5, 6]. The cytotoxic effect of BRD4 repression might be regulated in a NF- κ B dependent manner as BRD4 associates with NF- κ B. This hypothesis is further supported by the findings that the expression of the NF- κ B target gene BCL-2 is repressed upon BRD4 inhibition. It will therefore be interesting to verify whether the repression of BRD4 target genes such as BCL-2 and C-MYC is mediated via inhibition of the transcription factor NF- κ B.

In conclusion, we have shown that the NF- κ B pathway is essential for the survival of AML cells and that NF- κ B activity can be targeted in various ways. Future research will be necessary to validate the efficacy of candidate drugs that inhibit NF- κ B activity. Furthermore, more research will be required to dissect the exact role of the binding partners and coactivators of NF- κ B in transcription. Understanding the exact mechanism of these inhibitors could contribute to a better clinical application.



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